



ATPase activity and transport by a cGMP transporter in human erythrocyte ghosts and proteoliposome-reconstituted membrane extracts

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Abstract

We previously described the [^3H]cGMP-binding characteristics of a CHAPS-solubilized protein that we proposed to be a cGMP transporter. We now report the ATPase activity of the membrane-bound, solubilized and reconstituted form of a cGMP transporter. The membrane-bound protein of unsealed ghosts had a linear ATPase activity over a 120 min incubation period with optimal activity of about 400 pmol/mg/min. The apparent K_m and V_{max} for ATP were about 0.5 mM and 300 pmol/mg/min, respectively. When solubilized with CHAPS the specific activity of the protein was reduced to about 70 pmol/mg/min. Reconstitution of the CHAPS preparation into phospholipid bilayer using rapid detergent removal by Extracti-gel[®] column resulted in proteoliposomes which had ATPase activity similar to that found in the erythrocyte membranes. The proteoliposomes displayed a linear ATP-dependent uptake of [^3H]cGMP with an apparent K_m value of 1.0 μM . This low K_m -uptake of [^3H]cGMP in proteoliposomes was not affected by 10 μM of AMP, cAMP and GMP, but was completely abolished in the presence of the non-hydrolyzable ATP analogue, ATP- γ -S. Some ATPase activation was also observed in the presence of 2 μM cAMP, but it is unclear whether this activity was coupled to the cGMP transporter. Our results show that the membrane protein responsible for cGMP transport has an ATPase activity and transports the cyclic nucleotide in the presence of ATP. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: cGMP; ATPase; Membrane; Transport

1. Introduction

The existence of the intracellular signal molecule guanosine 3'5'-cyclic monophosphate (cGMP) in plasma and urine have been documented [1,2].

cGMP appears to be a biological marker since increased extracellular levels of cGMP in patients with heart failure [3–5] and with several types of neoplastic diseases including cancer of the lung, colon and breast [6] exist. Studies with different cell types like endothelial and vascular smooth muscle cells [7], hepatocytes [8,9] and human erythrocytes [10,11] have shown that cGMP egression is an energy-requiring process. The efflux of cGMP may serve not only the purpose of regulating the intracellular levels of cGMP, but the released cyclic nucleotide may also regulate the functions of other cells like the inhibition of transepithelial sodium transport in renal tubular cells [12]. Despite the significant role

Abbreviations: ATP, adenosine 5'-triphosphate; ATP- γ -S, adenosine 5'-O-(3-thiotriphosphate); CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate; cGMP, guanosine 3'5'-cyclic monophosphate; SDS, sodium dodecyl sulfate

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of adenosine 5'-triphosphate (ATP) in energizing cGMP transport [10,11], the ATPase activity of the transporter has yet not been characterized.

We previously demonstrated that the zwitterionic detergent 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfate (CHAPS) is capable of solubilizing and maintaining the cGMP-binding capacity of the putative transporter [13]. In the present study, we have made use of this information to isolate and reconstitute the human erythrocyte cGMP transporter into lipid bilayer model membranes, and characterize the cGMP-stimulated ATPase activities of the protein and transport of cGMP.

2. Materials and methods

Ammonium molybdate, disodium-ATP, ATP- γ -S, cGMP, GMP, cAMP, AMP, magnesium chloride, sodium dodecyl sulfate (SDS), sodium metaarsenite and L- α -phosphatidylcholine type II (from soybean) were from Sigma Chemicals (St. Louis, MO, USA). Extracti-Gel[®] D was purchased from Pierce Chemical Co. (Rockford, IL, USA). [³H]cGMP (specific activity 10 Ci/mmol) from Amersham International (Amersham, Buckinghamshire, UK). All other chemicals were of analytical grade.

2.1. Preparation of erythrocyte ghosts

Blood from healthy human donors was sampled into EDTA-vacutainer tubes and centrifuged at 600 $\times g$ for 15 min at 4°C. After removing plasma and buffy coat, the erythrocytes were resuspended in the KR bicarbonate buffer (NaCl 121 mM, KCl 4.8 mM, KH₂PO₄ 1.2 mM, NaHCO₃ 25.3 mM, CaCl₂ 1.3 mM, with a pH of 8.0 at 20–22°C) to restore the original hematocrit. The cells were washed and resuspended in the same buffer at 4°C. Unsealed membranes were prepared from washed erythrocytes using the method of Dodge [14] with slight modifications. The packed erythrocytes were lysed by resuspension in ice-cold deionized water and centrifuged at 3 \times 26 000 $\times g$ for 10 min. Membranes (milky white) were resuspended in solubilization buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.5 at 21°C).

2.2. Membrane solubilization

Solubilization of membrane proteins by 0.5% CHAPS (w/v) was performed using the method of Doige [15] with slight modifications. After shaking the membrane-detergent suspension at 4°C for 30 min, the mixture was centrifuged at 4°C for 1 h at 105 000 $\times g$. The resulting supernatant (S₁) was removed and discarded. The pellet was resuspended in the solubilization buffer to give a final protein concentration of 0.4–0.5 mg/ml and 0.5% CHAPS (w/v) was added. After 30 min incubation at 4°C, the sample was pelleted at 15 000 $\times g$ for 15 min at 4°C. The resulting supernatant (S₂) containing the cGMP transport protein as well as other proteins was removed carefully without pellet contamination, reconstituted into liposomes and assayed for its ATP hydrolytic and transport activities.

2.3. Proteoliposomes preparation

Proteoliposomes were prepared at 4°C according to the method of Horne [16] with certain modifications. CHAPS (1% w/v)-solubilized soybean phosphatidylcholine (130 μ l of a 50 mg/ml solution) was added to 400 μ l protein extract (S₂). MgSO₄ (54 μ l, 100 mM) was then added and the lipid-protein suspension was shaken for 30 min. After incubation, the mixture was applied to an Extracti-gel[®] column (1 ml) which has previously been treated with four volumes of 10 mM Tris-HCl, 150 mM NaCl buffer (pH 7.5) containing 2 mg/ml of bovine serum albumin and equilibrated with two volumes of 10 mM Tris-HCl, 150 mM NaCl, 10 mM MgSO₄ (pH 7.5). Elution of proteoliposomes from the column was done by using 1.5 ml of the equilibration buffer. The proteoliposomes were sedimented in a microcentrifuge at 14 000 $\times g$ for 10 min at 4°C and resuspended in 400 μ l of 10 mM Tris-HCl, 150 mM NaCl buffer, pH 7.5. For a typical ATP hydrolysis or [³H]cGMP uptake experiments, three of these resuspensions were employed.

2.4. Protein concentrations

Protein concentration was determined by a dye binding assay [17] using reagents from Bio-Rad Lab-

oratories (Richmond, CA, USA) and BSA from Sigma Chemical (St. Louis, MO, USA) as standards.

2.5. ATPase activity assay

ATPase activity of the transporter in erythrocyte ghosts, solubilized extract and its reconstituted form was determined by measuring the release of inorganic phosphate (P_i) from ATP, using the colorimetric method of Chifflet [18]. The experiment was performed in duplicate and each reaction medium of 200 μ l contained 10 mM Tris-HCl, 150 mM NaCl (pH 7.5), protein sample, 2 mM $MgCl_2$ (if not otherwise stated), 1 mM ATP (if not otherwise stated), 1 mM ouabain, and 2 mM EGTA with or without 2 μ M cGMP. After incubation at 37°C for up to 120 min, the reaction was stopped by the addition of 200 μ l of 12% SDS. Ascorbic acid 6% (400 μ l) in 1 M HCl/1% ammonium molybdate was then added for color development and the products stabilized after 10 min by adding 600 μ l of 2% sodium citrate/2% sodium metaarsenite/2% acetic acid. The absorbance of each sample at 850 nm was measured in Pharmacia LKB-Ultrospec III (Pharmacia LKB Biochrom Ltd, Cambridge CB4 4FJ, UK). The P_i released was calculated as the difference between incubations with and without cGMP.

2.6. [3H]cGMP uptake

The accumulation of cGMP into proteoliposomes was assayed with the help of [3H]cGMP. The experiment was performed in duplicate and each reaction medium of 500 μ l contained 10 mM Tris-HCl, 150 mM NaCl (pH 7.5), proteoliposomes, 2 mM $MgCl_2$ and 2 μ M [3H]cGMP (if not otherwise stated) with or without 1 mM ATP. After incubation at 37°C for up to 120 min, the reaction was stopped by adding 2 ml of ice-cold 10 mM Tris-HCl, 150 mM NaCl (pH 7.5) and immediately filtering through a Millipore cellulose filter type GS 0.22 which has previously been soaked in the same buffer. The filters were washed with 10 ml of 10 mM Tris-HCl, 150 mM NaCl (pH 7.5) and placed in scintillation vials containing 0.5 ml of 1.0 M HCl and 9.5 ml of scintillation liquid (Ultima Gold XR, Packard Instrument B.V., The Netherlands). Radioactivity was washed out of filters by gentle shaking for 20 h, and

measured in a Packard Tri-Carb scintillation spectrometer, Model 1900TR (Packard, Meridian, MS, USA). [3H]cGMP uptake was defined as the difference between accumulations with and without ATP.

2.7. Thin layer chromatography

The purity of the radiolabelled cGMP was examined with thin layer chromatography in three different systems: 0.3 M LiCl/PEI cellulose, 1.0 M KH_2PO_4 , pH 3.4/PEI cellulose and ethanol:1.0 M ammonium acetate (50:20)/Silica gel 60. In each system the radioactivity associated with the proteoliposomes was found in a single peak with the same R_f value as the native substance.

2.8. Statistics

The results are presented as mean value \pm S.D.

3. Results

3.1. cGMP stimulation of ATPase activity in unsealed ghosts

The transport system for cGMP has been demonstrated to be coupled to an ATPase since ATP, but not the non-hydrolyzable ATP analogues AMP-PNP and ATP- γ -S, supports cGMP transport [11]. In Fig. 1 (upper panel) we show that human erythrocyte membranes do possess intrinsic cGMP-stimulated ATPase activity. The production of free P_i from ATP was determined as the difference between incubations with and without cGMP. The rate of free P_i released was linear over a 120 min period and was found to be reproducible. The mean stimulated ATPase activity of three separate erythrocyte membrane preparations was 383 ± 27 pmol/mg/min. In spite of the fact that incubations contained Ca^{2+} and Na^+ , K^+ -ATPase inhibitors, EGTA and ouabain, respectively, an appreciably high level of basal ATPase activity was observed (278 ± 90 pmol/mg/min) in the absence of cGMP. Based on the data presented in Fig. 1 (middle panel), kinetic constants for the ATP hydrolysis in erythrocyte membranes from three separate experiments yielded an apparent K_m value for ATP of 0.48 ± 0.26 mM and a V_{max} value of 280 ± 70

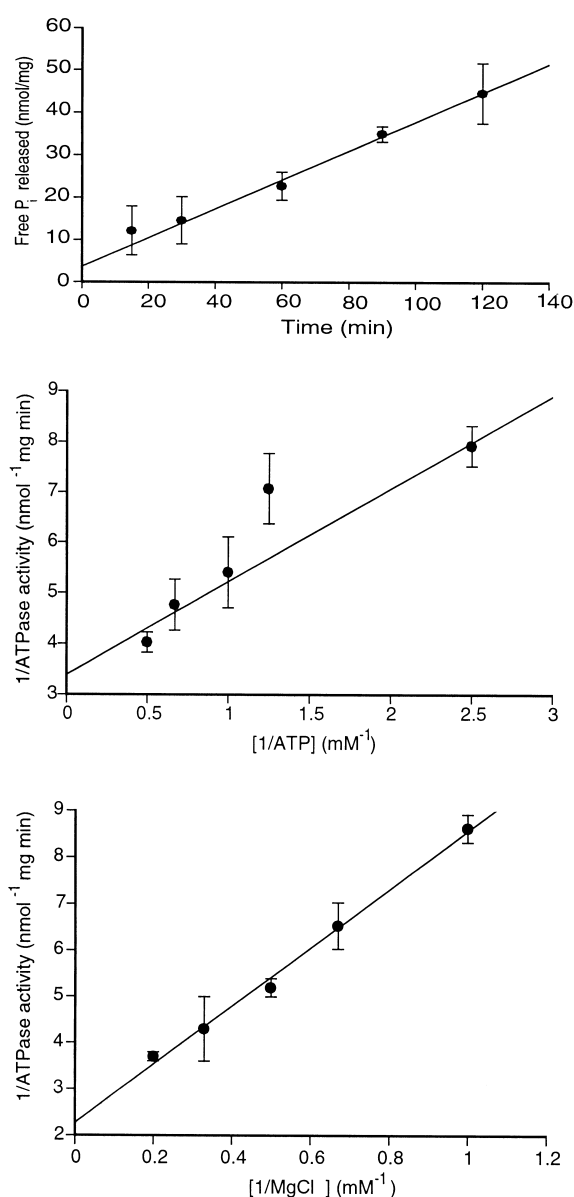


Fig. 1. cGMP-stimulated ATPase activity in unsealed erythrocyte ghosts. Human erythrocyte membranes (40–60 μ g) were incubated with and without 2 μ M cGMP in 200 μ l incubation buffer. After incubation at 37°C for up to 120 min, the ATPase activity was measured colorimetrically by following the production of P_i. P_i released is given as the difference between incubations with and without cGMP. Upper panel: Time course of cGMP-stimulated ATPase activity in unsealed erythrocyte ghosts. P_i released can be described by the linear correlation $y = 3.6 + 0.3x$ ($r = 0.98$). Results are presented as mean \pm S.D. ($n = 3$). Middle panel: ATP dependency of cGMP-stimulated ATPase activity in erythrocyte unsealed ghosts. Membranes were incubated with various concentrations of ATP (0.4–2 mM). After incubation for 120 min, the P_i released was measured and can be described by the linear correlation $y = 3.3889 + 1.8361x$ ($r = 0.98$). Results are presented in Lineweaver–Burk plot, mean value \pm S.D. ($n = 3$). Lower panel: Mg²⁺-dependency of cGMP-stimulated ATPase activity. Membranes were incubated with various concentrations of MgCl₂ (1–5 mM). After incubation for 120 min, the P_i released was measured and can be described by the linear correlation $y = 2.2686 + 6.2878x$ ($r = 0.99$). Results are presented in a Lineweaver–Burk plot, mean value \pm S.D. ($n = 3$).

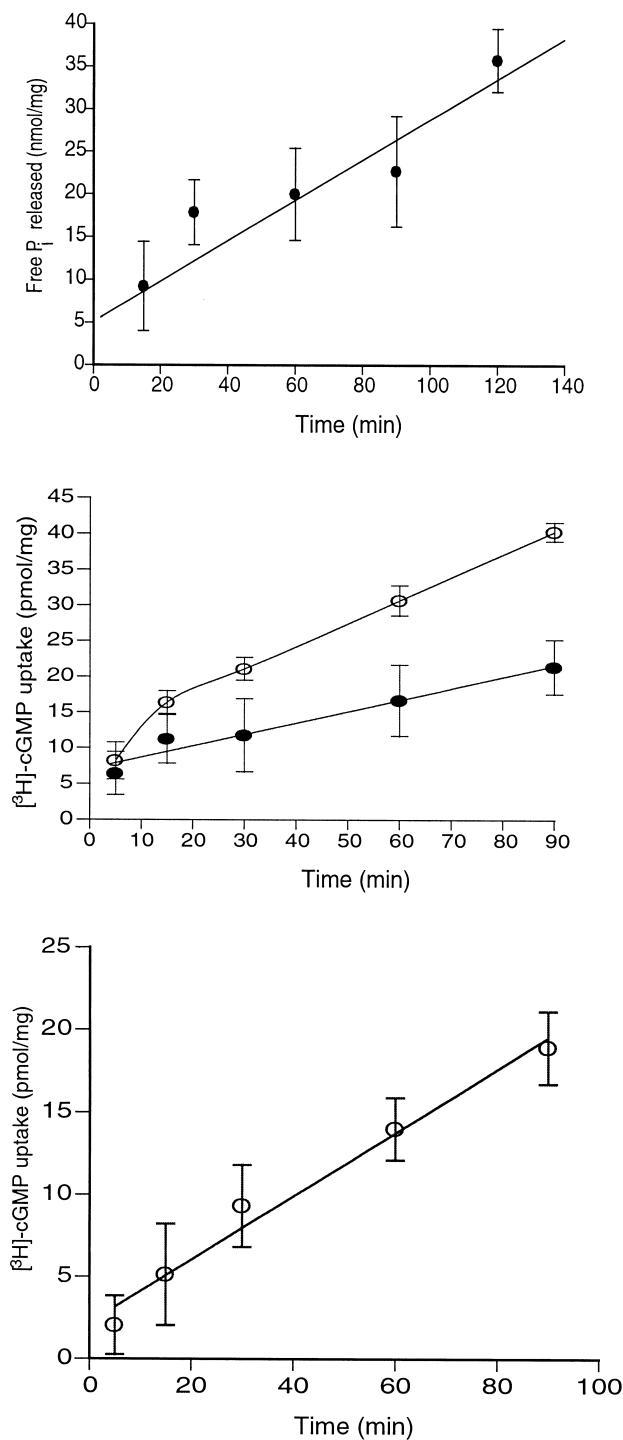
3.2. ATPase activity in solubilized extract

Solubilization of the erythrocyte membranes by 0.5% CHAPS (w/v) resulted in an extract with ATPase activity. The difference in P_i released between incubations with and without (2 μ M) cGMP is defined as cGMP-specific activity and showed linearity ($y = 0.29122 + 0.062875x$, $r = 0.99$). However, the rate of free P_i produced by the extract was about five times less than observed in the ghosts. The basal and stimulated ATPase activity of four distinct extracts were 21 ± 3 pmol/mg/min and 66 ± 16 pmol/mg/min (mean \pm S.D.), respectively.

3.3. ATPase activity and [³H]cGMP uptake in proteoliposomes

When detergent was removed and proteins reconstituted into liposomes, ATPase activity was raised to a level similar to that found in the unsealed ghosts. The background and stimulated ATPase activity of three separate proteoliposomes preparations were 257 ± 66 pmol/mg/min and 304 ± 35 pmol/mg/min, respectively. Fig. 2 (upper panel) shows a linear cGMP-specific ATPase activity up to 120 min at 37°C. [³H]cGMP accumulated in proteoliposomes

pmol/mg protein/min. The divalent cation, Mg²⁺, is a well-known activator of various ATPases [19] and has recently been reported to activate cGMP uptake into inside-out vesicles from human erythrocytes [20,21]. In the present study Mg²⁺ caused a concentration-dependent activation of the cGMP-stimulated ATPase activity (Fig. 1, lower panel). Under the present experimental conditions, the apparent K_m and V_{max} values for Mg²⁺ were 3.05 ± 0.9 mM and 520 ± 210 pmol/mg protein/min, respectively.



up to 90 min with and without ATP (Fig. 2, middle panel). The ATP-dependent uptake of cGMP (i.e. active transport of cGMP) in proteoliposomes, defined as the difference in accumulated [³H]cGMP in

Fig. 2. Re-established cGMP-stimulated ATPase activity and [³H]cGMP uptake in reconstituted erythrocyte membrane proteins (15 µg protein). Proteoliposomes were obtained as described in Section 2. Upper panel: Time course of ATPase activity in proteoliposomes incubated at 37°C for up to 120 min with and without 2 µM cGMP in the incubation medium described in Section 2. P_i released is given as the difference between incubations with and without cGMP. Results are presented as mean ± S.D. (n = 3). Middle panel: Time course of [³H]cGMP uptake in proteoliposomes at 37°C. Proteoliposomes were exposed to 2 µM [³H]cGMP in the presence or absence of 1 mM ATP in the incubation medium described in Section 2. Separation of proteoliposomes from medium was accomplished by dilution and filtration. [³H]cGMP uptake with (○-○) and without (●-●) ATP. Results are presented as mean ± S.D. (n = 3). Lower panel: Active [³H]-cGMP uptake is given as the difference between incubations with and without ATP (○-○) and can be described by the linear correlation $y = 2.1807 + 0.19245x$ ($r = 0.98$). Results are presented as mean ± S.D. (n = 3).

presence and absence of ATP, was linear (Fig. 2, lower panel). The mean uptake in three distinct proteoliposomes preparations was 214 ± 82 fmol/mg/min.

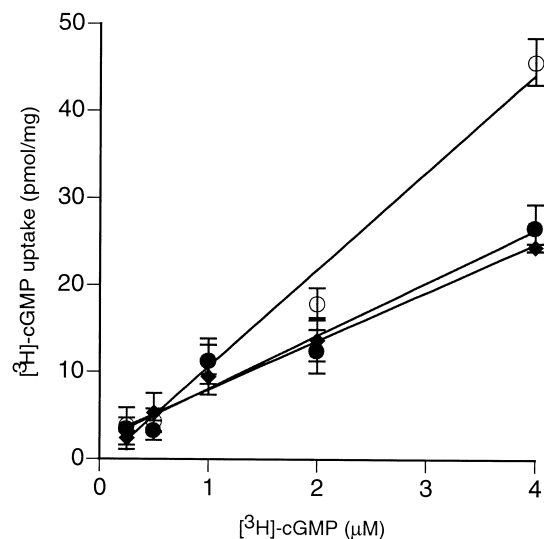


Fig. 3. The effect of ATP-γ-S on [³H]cGMP uptake in reconstituted erythrocyte membrane proteins (15 µg protein). Proteoliposomes were exposed to 0.2–4 µM [³H]cGMP in the presence and absence of 1 mM ATP or 1 mM ATP-γ-S. Separation of proteoliposomes from medium was accomplished by dilution and filtration. Results are presented as [³H]cGMP uptake with (○-○) and without (◆-◆) ATP and with (●-●) ATP-γ-S (mean ± S.D., n = 3).

We have previously shown that ATP, but not the non-hydrolyzable ATP analogue adenosine 5'-O-(3-thiotriphosphate) (ATP- γ -S), supports cGMP transport into inside-out vesicles from human erythrocytes [11]. Fig. 3 shows that [3 H]cGMP accumulated in proteoliposomes with ATP, ATP- γ -S, and also without ATP. The ATP-dependent uptake of cGMP was defined as the difference in accumulated [3 H]cGMP in presence and absence of ATP and it showed a concentration-dependent increase, whereas virtually no difference existed between [3 H]cGMP accumulation in the presence of ATP- γ -S and in absence of ATP. Scatchard analysis [22] of the data obtained from experiments with and without ATP (Fig. 3) gave an apparent K_m value of 1.05 ± 0.4 μ M.

3.4. Effects of nucleotides on ATP hydrolysis and [3 H]cGMP uptake in proteoliposomes

Stimulation of ATP hydrolysis in proteoliposomes by some nucleotides is presented in Table 1. cGMP-stimulated ATP hydrolysis was about fivefold that of cAMP whereas the non-cyclic nucleotides (AMP and GMP), had no stimulatory effect. The inhibition of [3 H]cGMP accumulation in proteoliposomes by these nucleotides is presented in Table 2. Among the nucleotides tested (AMP, cAMP, GMP, cGMP), only cGMP effectively inhibited the uptake of the radioligand by proteoliposomes. The other nucleotides did not show any inhibitory effect on [3 H]cGMP uptake.

Table 1
Effects of nucleotides on ATP hydrolysis by cGMP transporter

Nucleotide	Free P_i released (nmol/mg)
AMP	0
cAMP	4.4 ± 0.7
GMP	0
cGMP	23 ± 5

Proteoliposomes were incubated with and without 2 μ M of nucleotide (AMP, cAMP, GMP, or cGMP) in 200 μ l incubation buffer. After incubation at 37°C for 120 min, ATP hydrolysis was measured colorimetrically by assaying the production of P_i . P_i released is given as the difference between incubations with and without nucleotide. Results are presented as mean \pm S.D. ($n = 3$).

Table 2

Inhibition of [3 H]cGMP uptake in proteoliposomes by nucleotides

Nucleotide	Percent of control
AMP	115
cAMP	129
GMP	94
cGMP	0

Proteoliposomes were exposed to 2 μ M [3 H]cGMP, 2 mM $MgCl_2$ with and without 1 mM ATP in the presence or absence of 10 μ M nucleotide (AMP, cAMP, GMP or cGMP) at 37°C for 90 min. Separation of proteoliposomes from medium was accomplished by dilution and filtration. [3 H]cGMP uptake is defined as the difference in incubations with and without ATP. The results are presented as mean value of triplicate experiments.

4. Discussion

Direct biochemical evidence to demonstrate that cGMP transporter functions as an ATPase will be an invaluable support to the concept that the membrane protein utilizes ATP in energizing cGMP efflux. In this study we have shown the ATPase activity of a cGMP transporter in unsealed human erythrocyte membranes and made an initial characterization of the catalysis. Based on the evidence that ATPase activities of several transporter ATPases are directly regulated by the transported substrate, e.g., the Ca^{2+} - and Na^+/K^+ -ATPases [23], Cl^- -ATPase [24], the arsenite/antimonite ATPase [25], and the several organic molecule ATP-dependent transporters like the bacterial histidine and maltose permeases [26,27], the erythrocyte glutathione disulfide transporter [28] and P-glycoprotein (P-gp) [29], cGMP was used in stimulating the ATPase activity of the cGMP transporter.

Unsealed erythrocyte membrane exhibits cGMP-stimulated ATPase activity with a K_m value for ATP being comparable to that of other transporter ATPases, e.g., ArsA protein [25], CFTR [30], P-gp [15,29,31–33] and identical to the value obtained in cGMP transport studies [20]. The affinity of the transporter for the divalent cation, Mg^{2+} , is in the range reported for P-gp (2–10 mM) [31] and virtually similar to the K_m value observed for the activation of cGMP uptake into inside-out vesicles from human erythrocytes in a recent report [21]. CHAPS, unlike

other detergents, is known to preserve ATPase activity in proteins, e.g., P-gp [34] but reduces the activity by 50% or abolishes it completely when assayed in the presence of 1.6–9 mM of the detergent [29,31,32,34]. The low ATPase activity observed in the CHAPS-solubilized extract may be due to the effects of the detergent. Thus, the CHAPS might have caused delipidation and/or unfolding of the protein resulting in the disruption of the interaction between the substrate-binding site(s) and ATPase domain. The cGMP transporter, like other membrane-bound protein ATPases (e.g., Ca^{2+} - and $\text{Na}^{+}/\text{K}^{+}$ -ATPases, and P-gp), appears therefore to require a lipid environment for ATPase activity. Reconstitution of the transporter into liposomes made up of phosphatidylcholine, a phospholipid suitable for reconstitution and transport studies of erythrocyte membrane proteins [35–37], increased the ATPase activity to the level observed in membranes. This supports the concept mentioned above that the ATPase domain of the transporter requires stabilization of the transmembrane domain in a hydrophobic environment for its optimal activity.

We have successfully used reconstituted erythrocyte membrane extract to demonstrate cGMP uptake by a transport protein. The proteoliposome behaves qualitatively like cGMP transporter with respect to the use of ATP in translocating cGMP. Despite the fact that active cGMP uptake was linear (Fig. 2, lower panel), we could not estimate the stoichiometry of cGMP transported because this requires reconstitution of pure protein. In contrast to the observations for inside-out vesicles [10], the amount of [^3H]cGMP associated with the proteoliposomes in absence of ATP increased linearly (Fig. 2, middle panel). The fact that the apparent uptake of cGMP was identical in incubations with ATP- γ -S and without ATP (Fig. 3) demonstrates an ATP-independent process. This observation shows that the proteoliposome ‘membrane’ differs markedly from the cell membrane which is virtually impermeable to cGMP since concentration-gradients of 200 or more, is needed to load intact cells [38]. The proteoliposomes may have pores that allow diffusion into the vesicles or non-saturable binding (partition) to the artificial membrane.

The low K_m (1.0 μM) for [^3H]cGMP uptake is in agreement with our previous observations on active

cGMP transport across the human erythrocyte membrane [10,11,38]. If this active transport system obeys Michaelis–Menten kinetics, then saturation occurs at concentrations below 10 μM . In agreement, this concentration of unlabelled cGMP abolished the uptake of the tracer. On the other hand, identical concentrations of cAMP, AMP, and GMP did not hinder the uptake of [^3H]cGMP. This suggests a considerable degree of specificity in ranges of physiological concentrations. However, cAMP caused some ATPase activation and may reflect a loss of transport specificity at high cAMP concentration. In accordance, cAMP inhibited cGMP transport [11] with an estimated K_i of 0.8–3 mM. Another possibility is that the cAMP-induced ATPase activity is linked to a specific cAMP pump. Different profiles for the extrusion are observed for cGMP and cAMP [7,39] with cGMP showing a prolonged time of egress after the stimulation compared to an immediate rise and fall for cAMP. Furthermore, vinblastine, which is shown to reduce the export of cAMP [40] had no effect on the cGMP export [8]. However, cAMP transport shares at least some characteristics of cGMP transport, like the fact that the extrusion process is inhibited by probenecid [7,41,42]. Further studies are needed to clarify whether cAMP and cGMP share a common transporter.

The data of the present study strongly show that human erythrocyte membrane possesses an intrinsic cGMP transport system that utilizes ATP for its activity since no difference was evident in apparent [^3H]cGMP uptake without ATP and with the non-hydrolyzable ATP-analogue, ATP- γ -S. The need of ATP makes the cGMP transporter a potential member of the superfamily of ATP-binding cassette (ABC) transporters [43]. In agreement with this idea, MRP5 has recently been shown to transport cGMP and other nucleotides [44,45]. Most of the data we have accumulated on active cGMP transport [10,11,20,21,38] are consistent with MRP5 being the transport protein and include the cGMP pump in the family of ABC-transporters.

Acknowledgements

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